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# The gold-standard genome of *Aspergillus niger* NRRL 3 enables a detailed view of the diversity of sugar catabolism in fungi

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**Abstract:** The fungal kingdom is too large to be discovered exclusively by classical genetics. The access to omics data opens a new opportunity to study the diversity within the fungal kingdom and how adaptation to new environments shapes fungal metabolism. Genomes are the foundation of modern science but their quality is crucial when analysing omics data. In this study, we demonstrate how one gold-standard genome can improve functional prediction across closely related species to be able to identify key enzymes, reactions and pathways with the focus on primary carbon metabolism.

Based on this approach we identified alternative genes encoding various steps of the different sugar catabolic pathways, and as such provided leads for functional studies into this topic. We also revealed significant diversity with respect to genome content, although this did not always correlate to the ability of the species to use the corresponding sugar as a carbon source.

**Key words:** *Aspergillus*, Genomic diversity, Gold standard genome, Sugar catabolism.

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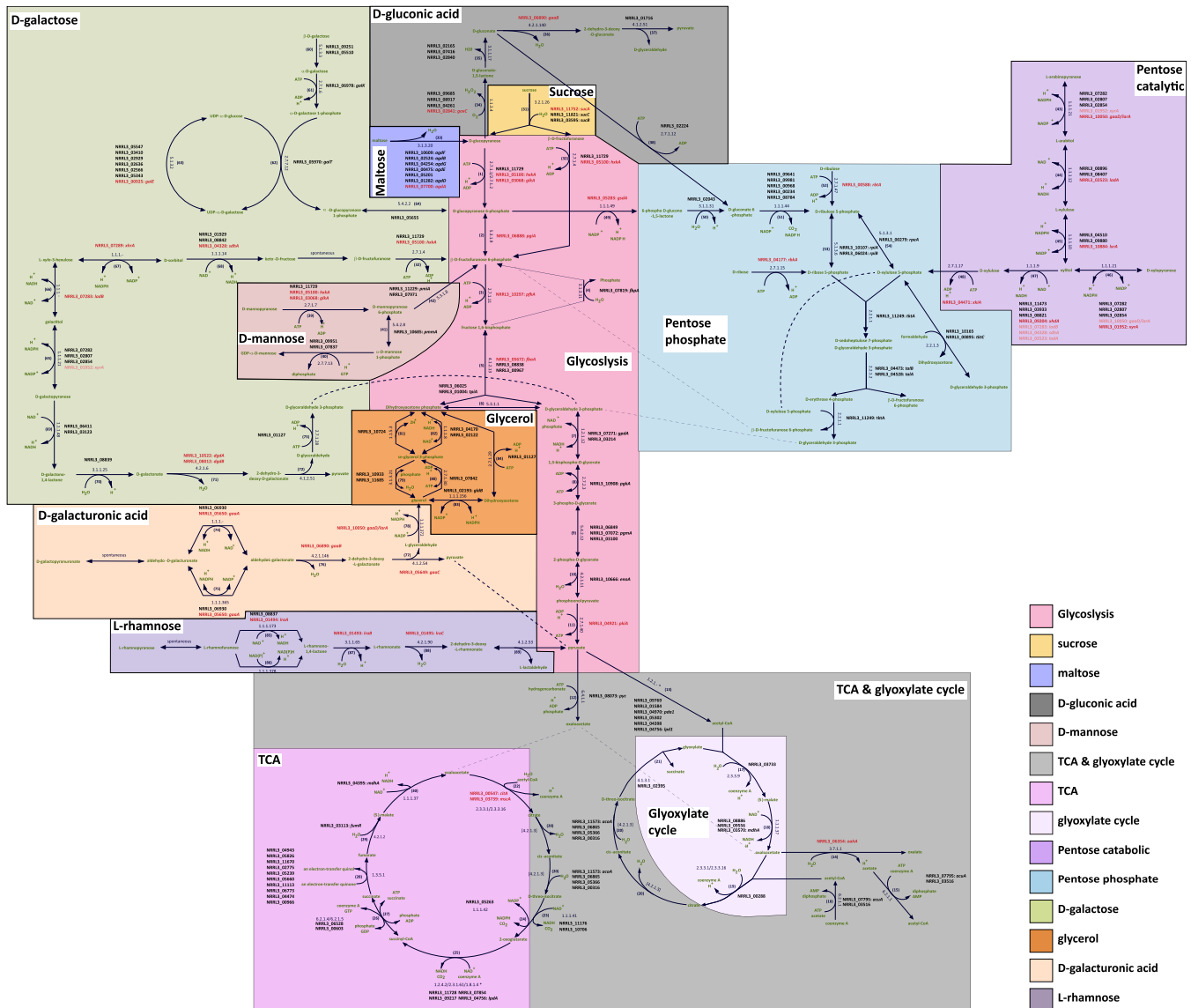
## INTRODUCTION

The fungal kingdom is estimated to contain over 1.5 million species (Hawksworth 1991), but only a few species have been studied in depth. In recent years, several hundreds of fungal genomes have been sequenced through different initiatives to gain insights into their biology and the variation within the kingdom (MIT, Arnaud *et al.* 2012, Grigoriev *et al.* 2012). Even though sequencing technology has evolved to produce the most complete genome sequence, we are still facing hurdles with respect to gene prediction and functional annotation (Aguilar-Pontes *et al.* 2014, Watson 2018). Recognizing genes in DNA sequences remains one of the most pressing problems in genome analysis together with the functional annotation of the predicted genes. This can only be improved using a large set of -omics data, literature and human supervision, also known as manual curation. If done properly, the output is a gold-standard genome that can be used to improve the quality of other genomes, especially of related species, to study the evolutionary mechanism that allow them to adapt to their lifestyles and ecological niches. The investments required to generate gold standard genomes go beyond what is feasible in a typical genome sequencing project, as it requires the combined efforts of not only sequencing centres, but also a broad research community that covers many of the biological aspects of fungal life.

Fungi and specifically Aspergilli, can be found in almost all ecosystems. In order to survive in their niches, they have to be able to accommodate their metabolism to the energy source available. In nature, fungi need to recognize the plant biomass

components in order to induce the production of the right set of degradative and metabolic enzymes that break down the complex structures forming the plant cell wall into simple molecules. Despite the complexity of the polymers forming the cell wall, the skeleton is mainly formed by simple sugars, such as D-glucose, D-fructose, D-xylose, L-arabinose, D-galactose, D-galacturonic acid and L-rhamnose (Kowalczyk *et al.* 2014). The ensemble of catabolic pathways that convert these sugars is known as primary carbon metabolism. A previous study investigated the evolution of primary carbon metabolism in *A. nidulans* and several Aspergilli based on the genome sequence available at that moment, identifying enzyme homologs and additional copies of several genes in some of the species (Flipphi *et al.* 2009). However, a recent study indicated that extra copies of an enzyme or a catabolic pathway does not necessarily affect the catabolic efficiency of the species under specific growth conditions, but correlates with the phylogenetic relationship between species (de Vries *et al.* 2017). However, a positive correlation was found between the presence of a gene predicted to encode a catabolic enzyme and their ability to grow on a specific carbon source (de Vries *et al.* 2017).

In this study, a network of reactions of primary metabolism based on the *Aspergillus niger* NRRL 3 gold-standard genome (Genozymes 2009) (manuscript in preparation) was generated and used to find orthologous genes and pathways involved in the catabolism of monosaccharides in a set of closely related fungal species (Fig. 1). The main focus was to evaluate whether there is a link between genome content and growth abilities. Our aim here is to study the genome content related to primary carbon



**Fig. 1. Primary carbon metabolism pathways:** summary overview of all pathways included in the *A. niger* NRRL 3 manually curated carbon metabolic network. Substrates are in green, reactions are depicted with an arrow, reversible reaction are indicated with double arrow. Enzyme Commission (EC) number for each reaction is indicated beside each reaction, while reactions are identified by numbers in brackets (for more information see [Supplementary file 6](#)). Proteins assigned to the reactions are indicated in black, common gene name used in *A. niger* is noted beside the protein ID where possible. Characterised enzymes are in red, enzymes involved in more than one reaction are indicated in lighter colour. Dash lines connect metabolites from different pathways. \* The enzymes associated to that reaction form a complex. Each pathway is highlighted with a background shade, the legend for the shades is on the right.

metabolism to identify key enzymes or reactions that explain growth under specific conditions. The advantage of using a gold-standard genome, with telomere-to-telomere chromosomes and manually curated gene models, as a reference for this study is that it significantly reduced the risk of missing genes due to gaps in the genome sequence and errors in electronic gene calling.

## MATERIALS AND METHODS

### Whole genome phylogeny

Whole genome phylogeny was performed among the 28 species selected (*Saccharomyces cerevisiae* S288C, *Neurospora crassa* OR74A, *Trichoderma reesei* QM 6a, *Talaromyces marneffei* ATCC 18224, *Talaromyces stipitatus* ATCC 10500, *Penicillium zonata* CBS 506.65, *Penicillium digitatum* PHI 26,

*P. chrysogenum* CBS 307.48, *P. rubens* Wisconsin 54-1255, *Aspergillus glaucus* CBS 516.65, *A. wentii* CBS 141173, *A. clavatus* NRRL 1, *A. novofumigatus* CBS 117520, *A. fumigatus* Af293, *A. fischeri* NRRL 181, *A. s. campestris* IBT 28561, *A. terreus* NIH 2624, *A. flavus* NRRL 3357, *A. oryzae* RIB40, *A. nidulans* FGSC A4, *A. sydowii* CBS 593.65, *A. versicolor* CBS 795.97, *A. aculeatus* ATCC 16872, *A. carbonarius* ITEM 5010, *A. brasiliensis* CBS 101740, *A. niger* NRRL 3, *A. luchuensis* CBS 106.47 and *A. tubingensis* CBS 134.48). The tree was built using 200 bidirectional best-blast hits (BBH), which were aligned using mafft V7.221 (Katoh & Standley 2013) with default parameters. Uninformative/ambiguous sites were removed from the alignment using Gblocks v0.91b (Nielsen et al. 1997) with parameters  $-t = p - e = .gb - b4 = 5$ . The maximum likelihood tree was built using FastTree v2.1.9 with parameters  $-\text{gamma} -\text{wag} .20$  rate categories were used during phylogeny reconstruction. This phylogenetic tree was visualized using R language and environment v.

**Table 1.** List of species used in this study.

Species	Strain	Section	Species abbreviation <sup>1</sup>	Number of genes	Assembly length (Mb)	Reference
<i>Saccharomyces cerevisiae</i>	S288C		Sacce1	6 575	12	(Goffeau <i>et al.</i> 1996)
<i>Neurospora crassa</i>	OR74A		Neucr2	10 785	41	(Galagan <i>et al.</i> 2003)
<i>Trichoderma reesei</i>	QM 6a		Trire2	9 143	33	(Martinez <i>et al.</i> 2008)
<i>Talaromyces marneffei</i>	ATCC 18224	Talaromyces	Talma1_2	10 638	29	(Nierman <i>et al.</i> 2015)
<i>T. stipitatus</i>	ATCC 10500	Talaromyces	Talst1_2	13 252	36	Unpublished
<i>Penicillium zonata</i>	CBS 506.65		Aspzo1	9 886	29	(de Vries <i>et al.</i> 2017)
<i>Penicillium digitatum</i>	PHI 26	Penicillium		9 118	29	(Marcet-Houben <i>et al.</i> 2012)
<i>P. chrysogenum</i>	CBS 307.48	Chrysogena	Pench1	11 396	31	(de Vries <i>et al.</i> 2017)
<i>P. rubens</i>	Wisconsin 54-1255	Chrysogena	PenchWisc1_1	13 671	32	(van den Berg <i>et al.</i> 2008)
<i>Aspergillus glaucus</i>	CBS 516.65	Aspergillus		11 277	28	(de Vries <i>et al.</i> 2017)
<i>A. wentii</i>	CBS 141173	Cremeri	Aspwe1	12 442	31	(de Vries <i>et al.</i> 2017)
<i>A. clavatus</i>	NRRL 1	Clavati	Aspcl1	9 121	28	(Fedorova <i>et al.</i> 2008)
<i>A. novofumigatus</i>	CBS 117520	Fumigati		11 549	32	(Kjaerbolting <i>et al.</i> 2018)
<i>A. fumigatus</i>	Af293	Fumigati	Aspfu1	9 781	29	(Nierman <i>et al.</i> 2005)
<i>A. fischeri</i>	NRRL 181	Fumigati	Neofi1	10 406	33	(Nierman <i>et al.</i> 2005)
<i>A. campestris</i>	IBT 28561	Candidi	Aspcam1	9 764	28	(Kjaerbolting <i>et al.</i> 2018)
<i>A. terreus</i>	NIH 2624	Terrei		10 406	29	(Arnaud <i>et al.</i> 2012)
<i>A. flavus</i>	NRRL 3357	Flavi		12 604	37	(Payne <i>et al.</i> 2006)
<i>A. oryzae</i>	RIB40	Flavi		12 030	38	(Machida <i>et al.</i> 2005)
<i>A. nidulans</i>	FGSC A4	Nidulantes	Aspnid1	10 680	30	(Galagan <i>et al.</i> 2005)
<i>A. sydowii</i>	CBS 593.65	Nidulantes	Aspsy1	13 620	34	(de Vries <i>et al.</i> 2017)
<i>A. versicolor</i>	CBS 583.65	Nidulantes	Aspve1	13 228	33	(de Vries <i>et al.</i> 2017)
<i>A. aculeatus</i>	ATCC 16872	Nigri	Aspac1	10 845	35	(de Vries <i>et al.</i> 2017)
<i>A. carbonarius</i>	ITEM 5010	Nigri	Aspca3	11 624	36	(de Vries <i>et al.</i> 2017)
<i>A. brasiliensis</i>	CBS 101740	Nigri	Aspbr1	13 000	36	(de Vries <i>et al.</i> 2017)
<i>A. niger</i>	NRRL 3	Nigri	Aspni_NRRL3_1	11 846	35	Unpublished
<i>A. luchuensis</i>	CBS 106.47	Nigri	Aspfo1	13 530	37	(de Vries <i>et al.</i> 2017)
<i>A. tubingensis</i>	CBS 134.48	Nigri	Asptu1	12 322	35	(de Vries <i>et al.</i> 2017)

The order of the species follows the taxonomic organization in the phylogenetic tree (Fig. 2).

<sup>1</sup> The species abbreviations correspond to JGI acronyms used as species and genome released unique identifiers in the portal. This identifier is used as species identifier in the heatmap figures.

3.4.0 (R Core Team 2017) with the package ggtree v. 1.8.2 (Yu *et al.* 2017) from ggplot2 v. 2.2.1 (Wickham 2009).

2012), Ensembl Fungi Biomart release 38 (Kinsella *et al.* 2011), (Benocchi *et al.* 2018) and the AspGD database (Cerqueira *et al.* 2014), respectively.

## Protein quality assessment

Protein quality assessment was performed using BUSCO v3 (Simao *et al.* 2015) with default parameters and specific lineage database accordingly (*Aspergillus*, *Penicillium*, *Penicillium* and *Talaromyces* species against *eurotiomycetes\_odb9* database, *N. crassa* and *T. reesei* against *sordariomycetes\_odb9* database and *S. cerevisiae* against *saccharomycetes\_odb9* database).

## Protein functional annotation

Protein functional annotation of selected species was downloaded from the JGI Mycocosm Portal (Table 1). Exclusively InterPro and Pfam domains (Quevillon *et al.* 2005) with E-value >1e<sup>-15</sup>, KEGG database information (Kanehisa *et al.* 2006) and SignalP annotation (Nielsen *et al.* 1997) was used. Additionally, *S. cerevisiae*, *N. crassa*, *T. reesei* and *A. nidulans* functional information was obtained from the SGD database (Cherry *et al.*

## Protein profiling

Gene families (clusters) were downloaded from JGI Mycocosm Portal (Grigoriev *et al.* 2014)

<https://genome.jgi.doe.gov/clm/run/fungi-2016-08.1352>. Clusters were predicted by Blast (Altschul *et al.* 1990) E-value 1e<sup>-5</sup> and MCL inflation parameter -2 (Enright *et al.* 2002). Clusters with only one protein assigned were removed from the original data set. In total, 112 gene clusters containing *A. niger* NRRL3 proteins of interest were selected. Amino acid sequences of selected species were downloaded from the JGI Mycocosm Portal (Table 1). Alignment of the amino acid sequences of the proteins included in the clusters was performed using mafft V7.221 (Katoh & Standley 2013) with default parameters. Alignments were manually curated and nucleotide sequences of split genes were fixed where possible, otherwise the protein sequence was removed from the alignment (Supplementary file



X1). A Neighbor joining tree was built using MEGA-CC (Kumar et al. 2012) with complete deletion and bootstrap 500. Phylogenetic trees were inspected manually and rooted to *S. cerevisiae* where possible. Proteins included in a monophyletic tree with an *A. niger* NRRL3 proteins will be considered as NRRL3 orthologs. Close orthologs of a NRRL3 protein will be defined as group of proteins included in a monophyletic tree after a gain or loss event where no NRRL3 protein is present. These are identified with the closest NRRL3 protein id and the suffix “like”. *A. niger* protein functional information was used to assign function to ortholog groups within a cluster. Groups with functional annotation not related to monosaccharides metabolism were removed from the final set when supported by more than one protein. If no NRRL3 protein was present in the group, curated database information of other species was used (Supplementary file X2). If more than one transcript per protein was included in the tree, they were counted as one (Supplementary file X3). Membership in a group was used to determine whether a protein was conserved across the selected species. If a protein is found at least once in all members or all members of an organism subset (e.g. section *Nigri*), it is considered conserved. A protein was considered specific to an organism subset if it was found in at least one organism of the subset, but not in any organisms outside the subset. Heatmaps were generated using R language and environment with the package ComplexHeatmap v. 1.14.0 (Gu et al. 2016). Columns are ordered according to the phylogenetic tree and rows follow the order of the reactions in the pathway. Growth graphs were generated using Adobe Illustrator CC.

## Growth profiling on different monosaccharides

For growth profiling, all strains were grown on MM (de Vries et al. 2004) with 33 different carbon sources (<http://www.fung-growth.org/>). Growth was performed at 30 °C for all *Aspergilli* and 25 °C for the other species. Growth was continued until the largest colony of a species set almost reached the edge of the plate. Growth per species was scored from 0 when there is no visible growth to 10 when maximum growth among all carbon sources has been reached. Growth profiles containing monosaccharides and disaccharides (de Vries, et al.) were selected for this study (Supplementary file X4). Media with no carbon source was used as a control. If growth on a specific carbon source is the same as with no carbon source, it is considered no growth.

## RESULTS

### Phylogenetic relationships and proteome assessment

To provide an overview of the relationship among the selected species (Table 1) a genome-wide phylogeny was conducted (Fig. 2A). The constructed tree supports the results described before (Kocsube et al. 2016). Members of the section *Nigri* are in a single clade close to section *Nidulantes* that also appears as a single clade. Section *Flavi* (*A. oryzae* and *A. flavus*) are placed close to the representative of section *Terri* with section *Candidi* as an outgroup. Section *Fumigati* and section *Clavati* are in another clade with *A. wentii* from section *Cremeri* as a close relative. The

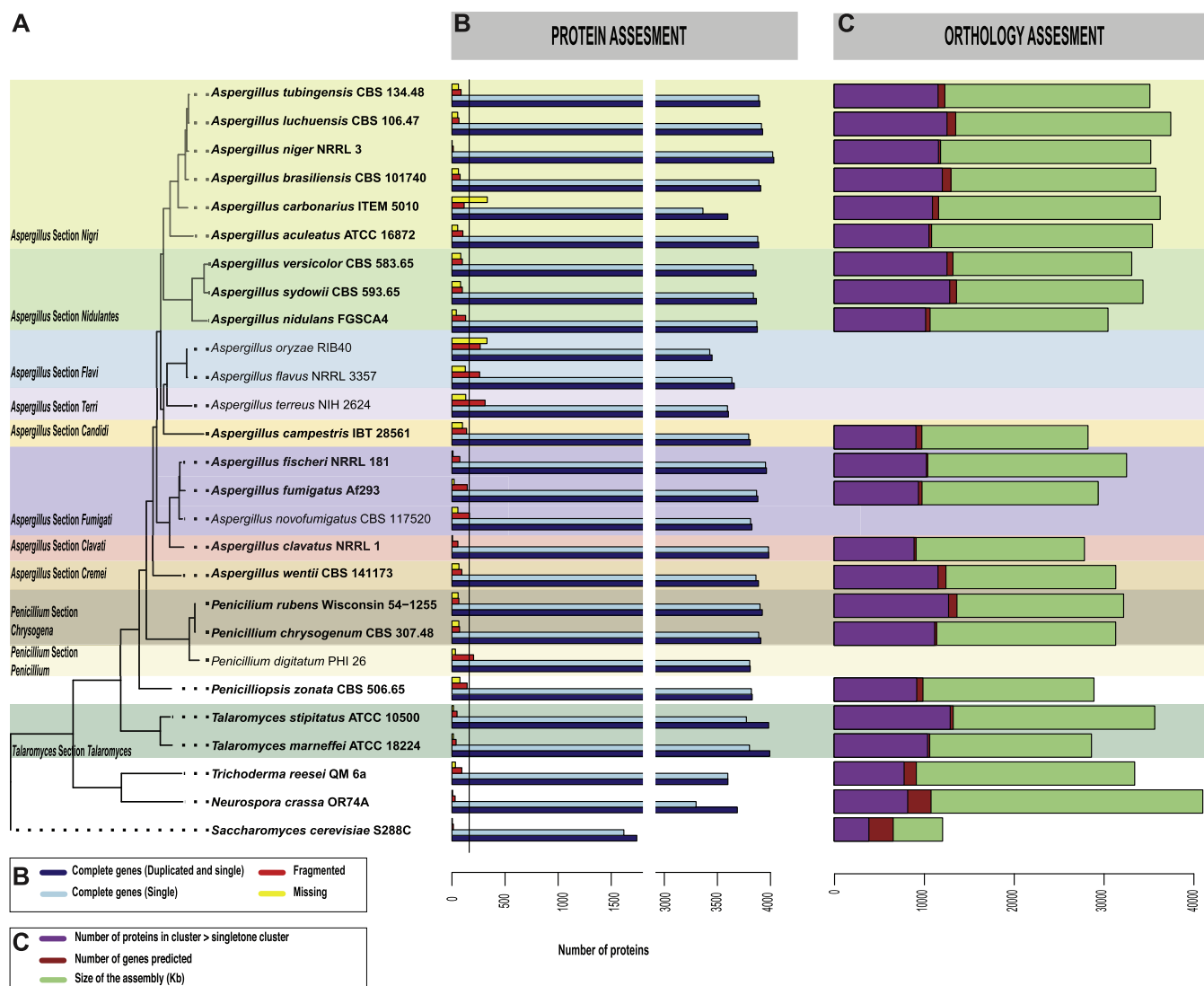
genus *Penicillium* sections *Chrysogena* and *Penicillium* are placed close to the related species *Penicillium zonata* (previously *Aspergillus zonatus* (Kocsube et al. 2016)). *T. reesei* and *N. crassa* are at the base of the tree with *S. cerevisiae* at the root.

The genomes used in this study have been sequenced over the years by different consortia and technologies. *S. cerevisiae* was the first fungal genome published in 1996 (Goffeau et al. 1996) while *A. campestris* was published in 2018 (Kjaerboling et al. 2018) sequenced with the latest technology PacBio RS. Hence the quality of assembled genomes and annotation can vary substantially. We used BUSCO (Simao et al. 2015) to evaluate the completeness across their predicted proteomes. The average of complete proteins is higher than 95 % in most species with the exception of *A. carbonarius*, where only 88 % of the genes are complete with more than 8 % of the core proteins missing. The proportion of the proteomes that are duplicated comprise approx. 1 % except for *S. cerevisiae*, *N. crassa*, *Talaromyces* species and *A. carbonarius* that varies between 5–10 %. With the exception of *A. carbonarius*, the genomes with the higher number of missing proteins also contain a higher number of fragmented proteins. High numbers of fragmented proteins were observed regardless the genus, technology, assembler or automatic gene calling methodology. Species with more than 150 fragmented proteins (*A. oryzae*, *A. flavus*, *A. terreus*, *P. digitatum*) were removed from the analysis (Fig. 2B).

Complete genomic sequences from diverse phylogenetic lineages reveal notable increases in genome complexity from prokaryotes to multicellular eukaryotes (Lynch & Conery 2003). However, the genome size of an organism varies from species to species and is not proportionally correlated with organismal complexity. In our dataset, the smallest genome is 12 Mb and the largest 41 Mb, corresponding to *S. cerevisiae* and *N. crassa* respectively, but the *N. crassa* genome only contains twice the number of genes of the *S. cerevisiae* genome. With the exception of *S. cerevisiae* and *N. crassa*, more than 95 % of the predicted proteins were included in the gene families downloaded from the JGI Mycocosm portal (Grigoriev et al. 2014) (Fig. 2C).

### Genome content related to primary carbon metabolism

In contrast to the most accepted theory (Gregory 2001), the present study shows that genome size and gene content are an indication of primary carbon metabolism complexity (Supplementary Fig. 5). In general, *S. cerevisiae* lacks several of the enzymes associated with primary carbon metabolism predicted in *A. niger*. In some cases, entire pathways are missing, even though growth has been described (Oliva Neto et al. 2014). Therefore, in the following sections we will describe the prevalence of orthologous genes in the different species without considering *S. cerevisiae* except for special cases. Starting with glycolysis and acid production (glyoxylate, tricarboxylic and D-gluconic acid) from glycolytic products, the pathways have been ordered according to the step in which the final product enters glycolysis and to the number of carbon atoms in the main substrate: maltose, sucrose, D-mannose, D-galactose, D-galacturonic acid, L-rhamnose, pentose catabolic and pentose phosphate pathway. In each case, a brief description of the pathway with the EC numbers of each enzymatic reaction and common protein names used in *A. niger* will follow a deep analysis of the relationship between phylogeny, genome content and growth



**Fig. 2. Phylogenetic tree, proteome assessment and orthology assessment.** **A)** Phylogenetic tree inferred from 200 best bidirectional hits of 28 species. Sections are identified by colours, white indicates the section is not determined. Name of the genus and sections are indicated in the left of the figure. Bold letters indicated the species selected for further analysis. **B)** Protein conservation, calculated using BUSCO v.3 indicates the degree of completeness of the proteins predicted per genome against a database of conserved proteins, bars showing number of proteins being aligned to individual species to the left-hand side. Black line indicates where the value 150 is in the graph. Species with number of fragmented proteins (red bar) higher than 150 were removed from the analysis. Dark blue: complete genes (duplicated and single), light blue: complete single genes, red: fragmented, and yellow: missing. **C)** Total number of proteins included in the gene family orthology against the total number of proteins predicted. Bars indicates the size of the genome (Kb), number of proteins in the proteome and number of proteins included in phylogeny aligned to individual species to the left-hand side. Green, size of genome (Kb); red, total proteome predicted; and purple, total number of proteins included in the gene families.

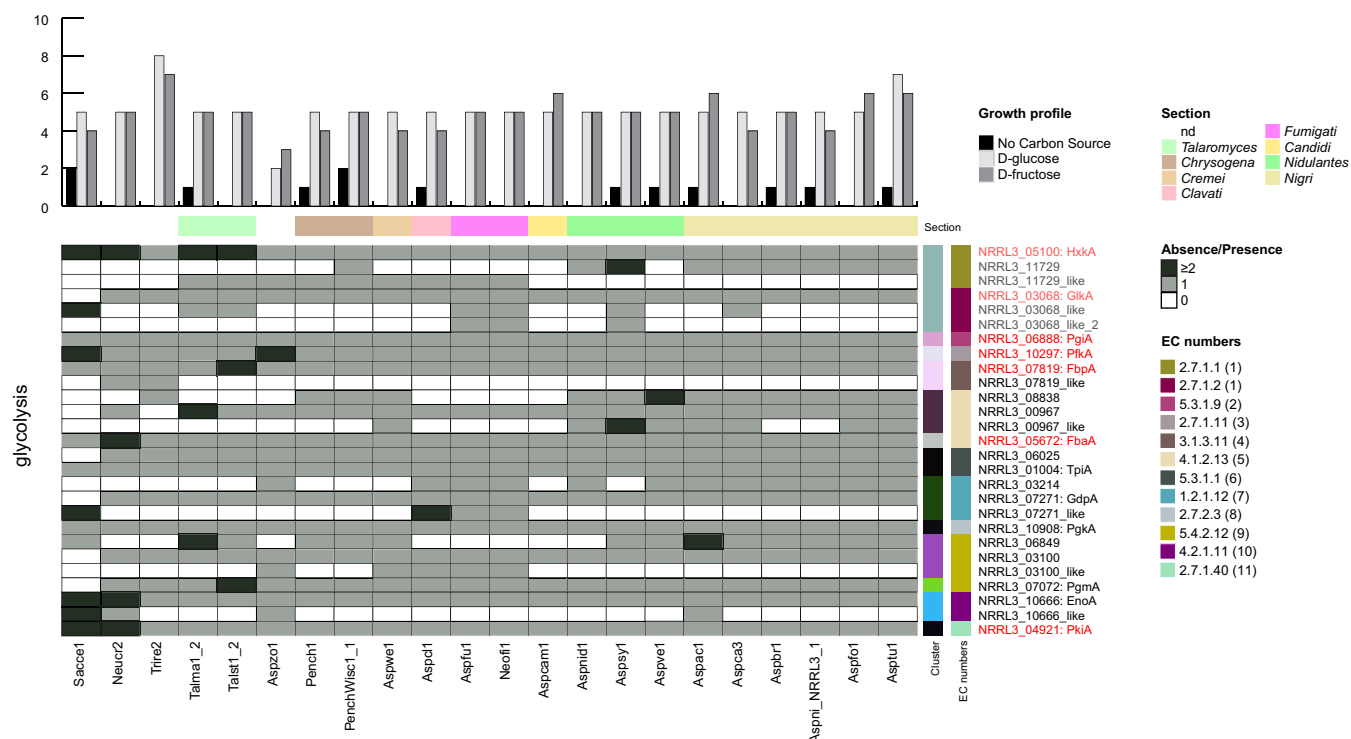
abilities. The ortholog groups will be referred to by the protein number of the *A. niger* NRRL 3 gene present in the group. If no NRRL 3 gene is present, the group will be referred to by the closest NRRL 3 paralog, followed by 'like'. Despite 'after' and 'before' not being the appropriate phylogenetic terms, we will use them to refer to moments in evolution when proteins have been gained or lost between two groups. Due to the limited number of species from each clade in this study we cannot determine when this exactly happened, e.g. in glycolysis, NRRL3\_11729\_like appears in members of the genus *Talaromyces* and is lost after section *Fumigati*. Meaning that species from clades after section *Fumigati* branched off, resulting in sections *Candidi*, *Nidulantes* and *Nigri* having lost this ortholog.

## Glucose and fructose catabolism

D-Glucose is the preferential monomeric carbon source for most microorganisms. Although fungi rarely find free high concentration of D-glucose in their environment, it is the major component

of the plant cell wall (Kowalczyk *et al.* 2014). Therefore, it is common that all species contain at least one enzyme per reaction for D-glucose catabolism (Fig. 3).

This first step in the glycolysis in the conversion of D-glucose into D-glucose 6-phosphate by two different enzymes, the hexokinase (Hxk, EC 2.7.1.1) (Panneman *et al.* 1998) and the glucokinase (Glc, EC 2.7.1.2) (Panneman *et al.* 1996). Isomerization of D-glucose 6-phosphate by glucose 6-phosphate isomerase (PfkA, EC 2.7.1.11) produces fructose 6-phosphate. This compound can be obtained from D-fructose through fructokinase reaction by hexokinase enzymes exclusively (Panneman *et al.* 1998). A kinase reaction catabolized by the 6-phosphofructose kinase (PfkA, EC 2.7.1.11) converts D-fructose 6-phosphate into D-fructose 1,6-bisphosphate (Habison *et al.* 1983). The reverse reaction, is catalysed by the D-fructose 1,6-phosphatase (FbpA, EC 3.1.3.11). Both reactions are strongly regulated by the accumulation of D-fructose 2,6-bisphosphate, which activates PfkA and inhibits FbpA (Harmsen *et al.* 1992, Ruijter & Visser 1999, Poulsen *et al.* 2005, Upadhyay & Shaw



**Fig. 3. Glycolysis heatmap** abundance of *A. niger* orthologous proteins in the glycolytic pathway. Columns are arranged according to the phylogenetic tree and rows are arranged according to the order in which the enzymatic reaction occur in *A. niger* metabolism. **Top:** The graph on the top represents growth from 0 to 10 with no carbon source (black), D-glucose (light grey) and D-fructose (dark grey). The line below represents the section to which the species belong according to the phylogenetic tree, colours match the colours in the tree. **Heatmap row names:** Identifiers on the right of the heatmap are according to the group name (see Materials and Methods). The name after the identifier correspond to the protein common name used in *A. niger*. Red: protein has been characterised in *A. niger*. Lighter colour: the protein is involved in more than one pathway. **EC numbers column:** colours correspond to EC number associated to the function they have been assigned. EC colour legend is on the right-hand of the figure. Reaction ID number are in brackets, see [Supplementary file 6](#) for more information. **Cluster column:** colours correspond to clusters in which proteins have been found. **Bottom legend:** species abbreviation according to [Table 1](#). Aspzo1: *Penicilliosis zonata*, Aspf1: *Aspergillus luchuensis*.

2006). D-Fructose 1,6-bisphosphate is split into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate by fructose-bisphosphate aldolase (Fba, EC 4.1.2.13) ([Jagannathan et al. 1956](#)). Dihydroxyacetone phosphate, also produced in glycerol metabolism, is converted into D-glyceraldehyde 3-phosphate by triphosphate isomerase (Tpi, EC 5.3.1.1) ([McKnight et al. 1986](#)) contributing to the equilibrium of the glycolysis. Glyceraldehyde 3-dehydrogenase (Gdp, EC 1.2.1.12) ([Punt et al. 1988](#)) catalyses the reversible enzymatic reaction between D-glyceraldehyde 3-phosphate and 1,3-bisphosphate D-glyceraldehyde, which is further converted into 3-phosphate D-glyceraldehyde by phosphoglycerate kinase (Pkg, EC 2.7.2.3) ([Clements & Roberts 1985](#), [Streatfield et al. 1992](#), [Streatfield & Roberts 1993](#)). Phosphopyruvate hydratase, also known as enolase (EnoA, EC 4.2.1.11) ([Clements & Roberts 1985](#), [Streatfield & Roberts 1993](#)), converts 2-phospho D-glycerate into phosphoenolpyruvate, the substrate of the last reaction in the glycolysis, the conversion of phosphoenolpyruvate into pyruvate by pyruvate kinase (PkiA, EC 2.7.1.40) ([de Graaff et al. 1992](#)).

Four out of ten enzymatic reactions in glycolysis - PgiA, PfkA, PkgA and PkiA - have a single, conserved enzyme assigned to them in all species (PgiA is present in more than one copy in *P. zonata* and PkiA in *S. cerevisiae* and *N. crassa*). A FbpA paralog was found through orthology in *N. crassa* and *T. reesei* while EnoA paralogs were only present in the genomes of *S. cerevisiae*, *N. crassa*, *P. zonata* and *A. aculeatus*.

Orthologs of HxkA and GlkA have undergone several changes in the number of proteins encoded in the genome of the tested species. Compared to a previous study ([Flipphi et al. 2009](#)), we found only one cluster containing a glucokinase and three hexokinases, out of which only two have catalytic function.

HxkA (NRRL3\_05100) is present in all species while its paralog, NRRL3\_11729 has two main groups. An orthologous gene present in *P. rubens*, *A. nidulans*, *A. sydowii* and section *Nigri* genomes and a close orthologous group that appears in members of the genus *Talaromyces* and got lost after section *Fumigati*. Three different Glk groups were identified in the phylogeny. An orthologous gene of *A. niger* GlkA (NRRL3\_03068) is present in all species. A second sequence (NRRL3\_03068\_like\_1) is present in the genome of the *Talaromyces* species, section *Fumigati*, *A. sydowii* and *A. carbonarius*. A third Glk protein (NRRL3\_03068\_like\_2) is also present in these species except for the *Talaromyces* species.

Proteins from two different clusters have been assigned to Fba activity. The ortholog of *A. nidulans* FbaA, NRRL3\_05672 (FbaA) is present in all species ([Roumelioti et al. 2010](#)) in a single cluster. The second cluster contain two orthologous groups NRRL3\_00967 and NRRL3\_08838, respectively. NRRL3\_00967 is present in all species except *T. reesei*, and a NRRL3\_00967\_like group is formed from genes of sections *Cremeri*, *Nidulantes* and several species from section *Nigri*. A similar distribution is observed for NRRL3\_08838, including members from the genus *Penicillium* and *T. reesei*. Iso-enzymes assigned to Pgm activity belong two different clusters. Both PgmA (NRRL3\_07072) and the second enzyme NRRL3\_03100 are present in all species. A NRRL3\_03100\_like orthologous gene was present in *P. zonata*, *A. clavatus* and section *Fumigati*.

Due to the importance of the pathway metabolizing D-glucose and D-fructose and the absence of orphan reactions, we predicted positive growth for all species on these sugars, which was confirmed by our growth data. *T. reesei* and *A. tubingensis* grew better on these substrates compared to other carbon sources





independent enzymes associated to them: the pyruvate dehydrogenase alpha and beta subunits (PdaA), the dihydrolipoamide S-acetyltransferase and the lipoamide dehydrogenase (LpdA). Another source of acetyl-CoA is the reaction catalysed by the acetyl-CoA synthetase (Acs, EC 6.2.1.1) from coenzymeA and acetate. The acetate used as substrate in this reaction can be provided by oxaloacetate acetylhydrolase (Oah, EC 3.7.1.1) after converting oxaloacetate into oxalate (Ruijter et al. 1999, Narayanan et al. 2009).

The glyoxylate and TCA cycles share three enzymatic reactions, with different paralogs for malate dehydrogenase and citrate synthase, but the same enzyme for aconitase.

Malate dehydrogenase (Mdh, EC 1.1.1.37) catalyses the reversible conversion between malate and oxaloacetate (McAlister-Henn et al. 1995). Oxaloacetate is converted into citrate by citrate synthase (Cit, 2.3.3.1). The citrate produced in the glyoxylic cycle is transported to the mitochondria to be converted first into cis-aconitate and finally to isocitrate (D-threo-isocitrate) by aconitate hydratase (Aco, EC 4.2.1.3) that catabolizes both steps. Isocitrate relocates to the peroxisome to continue the glyoxylate cycle.

In the peroxisome, during the glyoxylate cycle, isocitrate lyase (EC 4.1.3.1) will breakdown isocitrate into glyoxylate and succinate, which then enter the TCA. Finally, glyoxylate will be used as substrate together with acetyl-CoA for malate synthase (EC 2.3.3.9) to produce malate and coenzymeA.

In the mitochondria, during the TCA cycle, isocitrate is converted into 2-oxoglutarate by two different isocitrate dehydrogenase reactions, with different cofactors (NADP, EC 1.1.1.42 and NAD, EC 1.1.1.41). The 2-oxoglutarate complex converted coenzymeA to 2-oxoglutarate to produce succinyl-CoA. This complex requires three different enzymes, a mitochondrial 2-oxoglutarate dehydrogenase (EC 1.2.4.2), a dihydrolipoamide succinyl-transferase (EC 2.3.1.61) and the dihydrolipoamide dehydrogenase (EC 1.8.1.4). Succinate-CoA ligases (GTP as cofactor, EC 6.2.1.4 and ATP as cofactor 6.2.1.5) catabolize the conversion of succinyl-CoA into succinate. Succinate dehydrogenase (EC 1.3.5.1) oxidizes succinate into fumarate. Finally, the last step in the TCA cycle is the conversion of fumarate into malate by the fumarate hydratase (FumR, EC 4.2.1.2).

Most of the enzymatic reactions have multiple isoenzymes assigned to them. Only PycA (NRRL3\_08073), the peroxisomal malate synthase (NRRL3\_03733), isocitrate lyase (NRRL3\_02395) and the mitochondrial FumR (NRRL3\_03113) and MdhA\_M (NRRL3\_04395) are present as a single enzyme in all genomes, some of them are present as more than one copy.

Within the pyruvate dehydrogenase complex, pyruvate dehydrogenase has two subunits, alpha and beta. The alpha subunits, PdaA (NRRL3\_04970) and NRRL3\_09769 belong to the same cluster. PdaA is present in all species except in *A. nidulans* while NRRL3\_09769 is missing in *N. crassa*, *T. reesei* and *P. zonata*. The beta subunit NRRL3\_01584, belong to a different cluster containing orthologs for all species. Enzymes from two different clusters have been assigned to dihydrolipoamide S-acetyltransferase activity, with the *A. niger* genes NRRL3\_05302 and NRRL3\_04398 assigned to them, and orthologs for both are present in all species. The last component of the complex, LpdA (NRRL3\_04756), is present in all species. Acetyl-CoA synthase, AcsA (NRRL3\_07795) is also present in all species, but its paralog (NRRL3\_03516) is missing in *A. aculeatus*. A third enzyme, NRRL3\_03516\_like is present in *A. wentii*, *A. fischeri*, *A. campestris*, *A. versicolor*, *A. aculeatus* and *A. tubingensis*. Even

though in the network only one protein was assigned to the oxaloacetate dehydrogenase reaction, OahA (NRRL3\_06354), the orthology analysis shows two additional paralogous groups (NRRL3\_04875 and NRRL3\_06354\_like|NRRL3\_04875\_like). All genomes contain at least one protein assigned to this reaction. After *T. stipitatis* branched off multiple sequences are found reaching the higher number of paralogs in section *Nigri*.

In glyoxylate cycle, three isoenzymes have been assigned to the malate dehydrogenase reaction belonging to two different clusters. MdhA\_P (NRRL3\_03570) is present in all species, except *A. campestris*. A second enzyme in the same cluster, NRRL3\_09556 is present only in section *Nigri*, *A. nidulans*, *A. wentii* and the *Penicillium* species. The third group of enzymes from a different cluster, orthologs of NRRL3\_08886, is present in all species except *A. luchuensis* and *A. fischeri*. A NRRL3\_08886\_like group containing genes from *A. wentii*, section *Nidulantes*, *A. carbonarius* and *A. tubingensis* was also identified.

Initially, two citrate synthases assigned in the network were identified, NRRL3\_02449 and NRRL3\_11764, but according to *in house* data they do not participate during glyoxylate production. Nevertheless, A third paralog was identified through orthology. NRRL3\_00288 appeared after genus *Talaromyces* split off and it is only missing in *A. aculeatus*.

In the TCA cycle, citrate synthase is assigned to two mitochondrial enzymes, CitA (NRRL3\_00547) and McsA (NRRL3\_03739) that belong to the same cluster and are present in all species. NADPH<sup>+</sup>-dependent isocitrate dehydrogenase (NRRL3\_05263) is also present in all species. This cluster also contains NRRL3\_05263\_like sequences from *S. cerevisiae* and *A. campestris* that group separately. NADH<sup>+</sup>-dependent isocitrate dehydrogenase isoenzymes (NRRL3\_10706 and NRRL3\_11176) that belong to the same cluster, are present in all species.

Interestingly, one enzyme of each component of the 2-oxoglutarate dehydrogenase complex is present in almost all species, NRRL3\_07854, NRRL3\_09217 (missing in *T. marneffeii*) and LpdA (NRRL3\_04756). A second 2-oxoglutarate dehydrogenase NRRL3\_11728 and a dihydrolipoamide S-succinyl transferase NRRL3\_11727 are only present in *A. wentii*, *A. versicolor* and some members of section *Nigri*.

Succinate-CoA ligases NRRL3\_06528 and NRRL3\_00603 belong to different clusters. Both enzymes are present in all species. In addition, a NRRL3\_06528\_like group contains a *T. reesei* and *A. sydowii* sequence, and these species also contain a NRRL3\_00603\_like sequence together with *P. zonata*, *Penicillium* species, *A. wentii*, *A. clavatus*, species from section *Fumigatus* and *A. campestris*.

Succinate dehydrogenases assigned in the network belong to several clusters. All species contain between six and eight paralogs encoded in their genome, section *Nigri* contain up to ten paralogs.

Beside production of different acids, the intermediate metabolites of both cycles are used to produce different amino acids essential for the fungus to survive in the environment. Since growth on the pyruvate precursors D-glucose and D-fructose was positive, we can predict that all species have functional TCA and glyoxylate cycles.

## D-Gluconate metabolism

In the presence of D-glucose, some fungi will produce gluconate and its acid form D-gluconic acid as the primary overflow metabolite under non-limiting growth on glucose (Shindia et al.

2006) (Fig. 5). *A. niger* oxidizes D-glucose to D-glucono-1,5-lactone by a glucose oxidase from the carbohydrate active enzyme (CAZy) family AA2 (Lombard *et al.* 2014) (Gox, EC 1.1.3.4). D-glucono-1,5-lactone lactonodehydratase or gluconolactonase (EC 3.1.1.17) converts D-glucono-1,5-lactone to D-gluconate, which can be dehydrated to 2-keto-3-deoxy-D-gluconate by D-galactonate dehydratase (GaaB, EC 4.2.1.146) that is also involved in D-galacturonic acid metabolism. This is further cleaved to D-glyceraldehyde and pyruvate by 2-dehydro-3-deoxy-D-gluconate D-glyceraldehyde-lyase (EC 4.1.2.51). D-gluconate can enter the pentose phosphate pathway through the conversion to D-gluconate-6-phosphate catalysed by gluconokinase (EC 2.7.1.12).

Overall, genomes from sections *Nigri*, *Nidulantes* and genus *Penicillium* contain several predicted proteins associated to the reactions above, explaining the higher amount of gluconic acid production reported for these fungi (de Vries *et al.* 2017). They seem to have gained additional copies of glucose oxidase (Gox), after *N. crassa* diverged from the other species. The highest number of enzymes is observed for section *Nigri* with five Gox enzymes belonging to a single cluster, including the characterised GoxC (NRRL3\_02841). The high number of isoenzymes might suggest production of alternatives for lactonate, but no other enzymatic products have been described despite the broad use of GOX in industrial processes (Malherbe *et al.* 2003). Two gluconolactonases, belonging to different clusters, were predicted but the iso-enzyme NRRL3\_07416 is only present in section *Nigri*. A second enzyme belonging to GaaB cluster was present in many species outside the Aspergilli, suggesting it got lost after the Aspergilli branched off the other fungi. A similar situation was detected for 2-dehydro-3-deoxy-D-gluconate D-glyceraldehyde-lyases. Only one enzyme for this function was associated to the reaction in the network (NRRL3\_01716), but through orthology, we were able to identify eight different paralogous groups belonging to one of the largest clusters resulting from the orthology analysis. As mentioned above, there is a connection between D-gluconate and the pentose phosphate pathway not involving glycolysis for which NRRL3\_02224, the only enzyme assigned is present in all species, including *S. cerevisiae*.

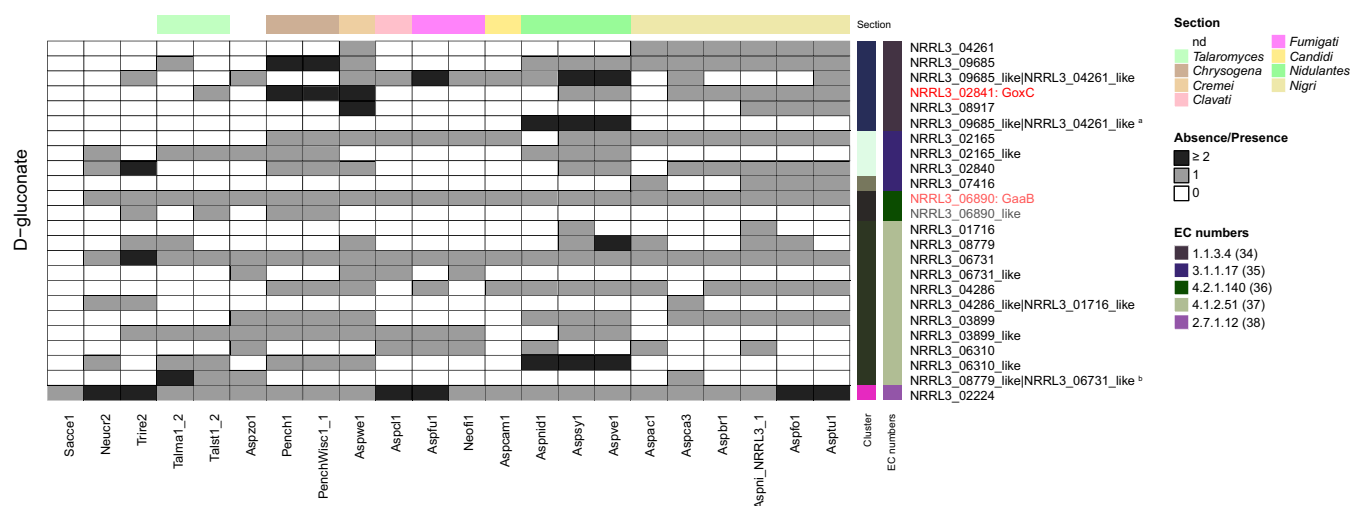
## Metabolism of the disaccharides maltose and sucrose

Maltose is a disaccharide formed by two molecules of  $\alpha$ -D-glucose with a 1,4-glucoside linkage. Through hydrolysis,  $\alpha$ -glucosidase produces two molecules of D-glucose that enters glycolysis.  $\alpha$ -glucosidase or maltase (EC 3.2.1.20) belongs to CAZy families GH13 and GH31 (Lombard *et al.* 2014) (Fig. 6). Seven different *A. niger* proteins have been assigned in the network. Enzymes from family GH31, AgdA (NRRL3\_07700), AgdB (NRRL3\_02524), AgdE (NRRL3\_00475), AgdF (NRRL3\_10609) and AgdG (NRRL3\_04254) belong to four different clusters (Kimura *et al.* 1992, Nakamura *et al.* 1997). Enzymes from family GH13, AgdD (NRRL3\_01282) and NRRL3\_05201 belong to a unique cluster. All species contain more than one protein in their genome from family GH31 from different clusters. Within family GH13, NRRL3\_05201 ortholog is present in all species of the Eurotiales. The NRRL3\_01282\_like group is mostly missing in section *Nigri*, while AgdD (NRRL3\_01282) is specific for section *Nigri*.

All species in this study contain more than one  $\alpha$ -glucosidase in their genome, which match their ability to grow on maltose as sole carbon source. *T. stipitatus*, *A. fischeri* and *A. niger* grew better on maltose than on D-glucose. *S. cerevisiae* is not able to grow on D-maltose which correlates with the presence of only two  $\alpha$ -glucosidases in its genome, AgdE and AgdD orthologs. Other than this example, there is no correlation between the number of enzymes encoded and their ability to grow on maltose.

Sucrose is a disaccharide of D-glucose and D-fructose and its hydrolysis is catalysed by  $\beta$ -D-fructofuranoside fructohydrolase (EC 3.2.1.26) belonging to CAZy family GH32 (Lombard *et al.* 2014), also known as invertase (Fig. 7). The catalytic reaction releases two monomers, an  $\alpha$ -D-glucose and a  $\beta$ -D-fructose that both enter glycolysis through the enzymatic reactions catabolized by hexokinases (HxkA and NRRL3\_11729) and glucokinases (Glk), where the latter only acts on  $\alpha$ -D-glucose.

There are three invertases (SucA-C) assigned to *A. niger* sucrose degradation (Boddy *et al.* 1993, Wallis *et al.* 1997), all of which belong to the same cluster. Invertases SucB (NRRL3\_03595) and SucC (NRRL3\_11821) appeared firstly in *T. stipitatus* genome. SucA (NRRL3\_11752) appeared when *Penicillium* species



**Fig. 5. D-gluconate heatmap** abundance of *A. niger* orthologous proteins in the D-gluconate catabolic pathway. The legends are the same as that of Fig. 3. Full group name: <sup>a</sup> NRRL3\_09685\_like, NRRL3\_04261\_like, NRRL3\_02841\_like, NRRL3\_08917\_like and <sup>b</sup> NRRL3\_08779\_like, NRRL3\_06731\_like and NRRL3\_05649\_like.

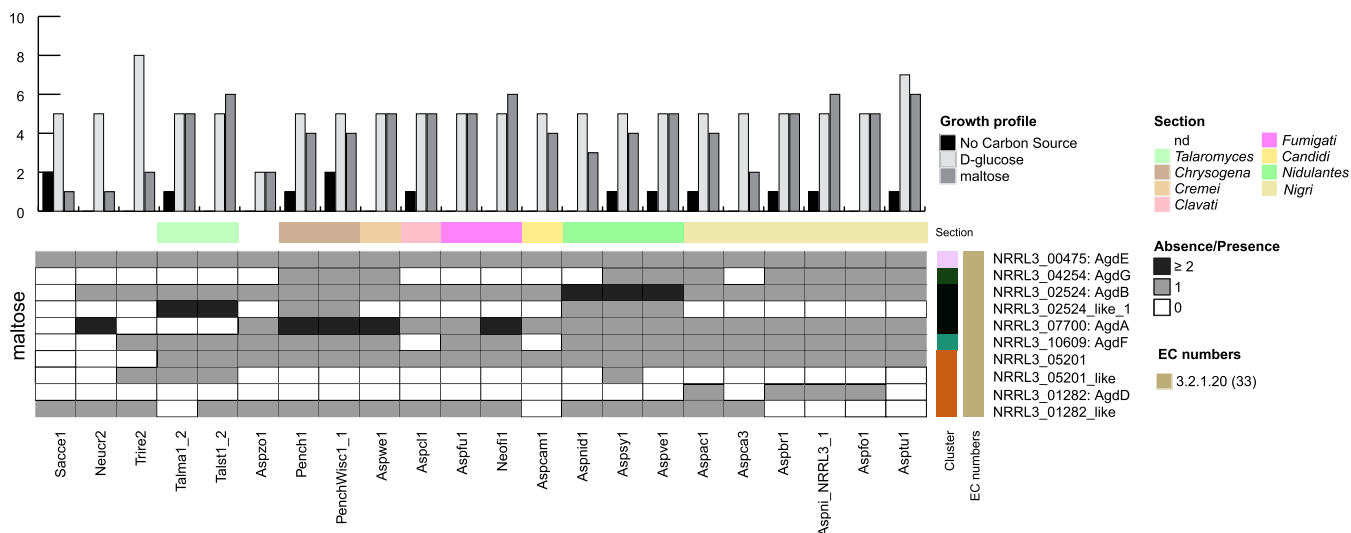


Fig. 6. Maltose heatmap abundance of *A. niger* orthologous proteins in the maltose catabolic pathway. The legends are the same as that of Fig. 3.

branched off. SucC is only present in *T. stipitatis*, *Penicillium* species, *A. brasiliensis* and *A. niger*, while SucB is present in all species except *P. zonata*, *A. clavatus* and *A. campestri*. SucA is missing in *A. clavatus*, *A. campestri*, *A. nidulans* and section *Fumigati*. In summary, *P. zonata*, *A. clavatus* and *A. campestri* lack all three enzymes, section *Fumigati* only contains SucB, section *Nidulantes* and *Nigri* contain mainly SucA and SucB, while *Penicillium* species contain all three.

All species show different degrees of growth on sucrose. *T. stipitatus*, *A. wentii*, *A. brasiliensis* and *A. luchuensis* grew better on this disaccharide than on the monosaccharides, D-glucose and D-fructose. *T. marneffei* showed reduced growth on sucrose compared to D-glucose and D-fructose. *Trichoderma reesei* is known for lacking invertases in its genome; however, it is able to grow when invertases from other species are heterologously produced (Berges et al. 1993), which corroborates the use of the resulting monosaccharides as substrates of glycolysis. On the other hand, *S. cerevisiae* and *N. crassa* showed growth on sucrose although they miss all three orthologous genes identified in *A. niger* NRRL 3, which showed the sequence diversity within the CAZy family or a different mechanism to degrade sucrose.

## D-Mannose metabolism

$\alpha$ -D-Mannose can be metabolized through glycolysis or utilized for the formation of glycoproteins through the production of GDP-

$\alpha$ -D-mannose (Fig. 8).  $\alpha$ -D-mannose is converted to  $\alpha$ -D-mannose-6-phosphate by Hxk (see glycolysis metabolism section) and it is linked to glycolysis through mannose-6-phosphate isomerase (PmiA, EC 5.3.1.8) (Ruijter & Visser 1999, Upadhyay & Shaw 2006) that converts D-mannose 6-phosphate in D-fructose-6-phosphate.

To produce GDP- $\alpha$ -D-mannose, phosphomannomutase (PmmA, EC 5.4.2.8) converts D-mannose into  $\alpha$ -D-mannose 1-phosphate that is further converted by the mannose-1-phosphate guanylyltransferase (EC 2.7.7.13) to GDP- $\alpha$ -D-mannose.

PmiA (NRRL3\_11229) and its ortholog NRRL3\_07971 and PmmA (NRRL3\_10685) are present in all species. Two clusters encoding mannose-1-phosphate guanylyltransferase were found. All species contain enzymes from these two clusters (NRRL3\_07837 and NRRL3\_09951) except *A. carbonarius* that contains also a NRRL3\_07837\_like protein.

In general, all species show growth on D-mannose similar to D-glucose, except *T. marneffei* that showed highly reduced growth compared to D-glucose.

## D-Galactose metabolism

In *A. niger* D-galactose can be metabolized through three different pathways (Fig. 9). The Leloir pathway and the oxidoreductive pathway final products of which enter glycolysis as D-fructose 6-phosphate or D-glucose 6-phosphate, and the non-

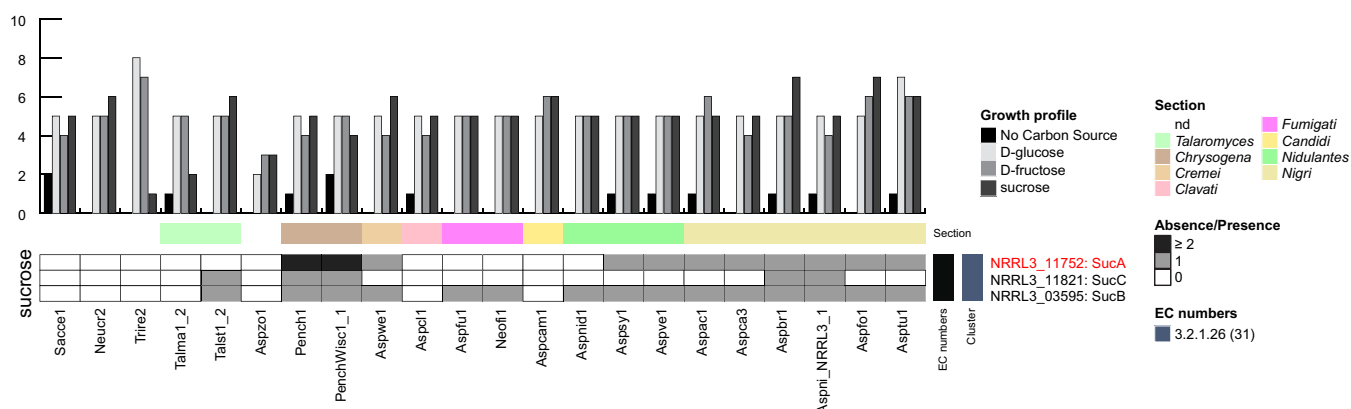


Fig. 7. Sucrose heatmap abundance of *A. niger* orthologous proteins in the sucrose catabolic pathway. The legends are the same as that of Fig. 3.

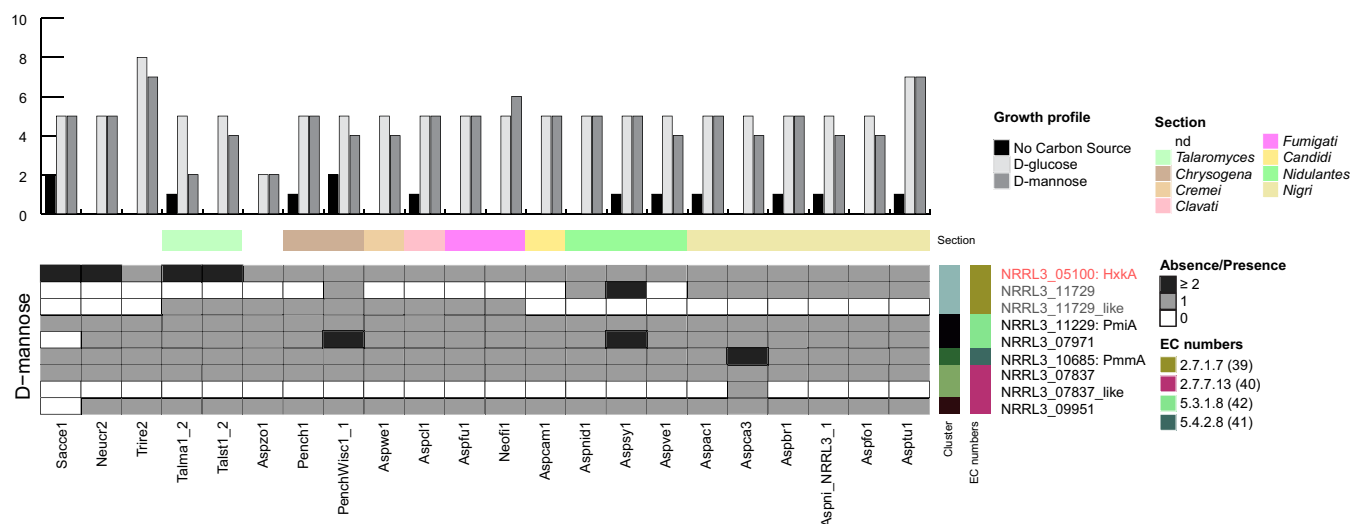


Fig. 8. D-mannose heatmap abundance of *A. niger* orthologous proteins in the D-mannose catabolic pathway. The legends are the same as that of Fig. 3.

phosphorylated DeLey-Doudoroff pathway whose final products are pyruvate and D-glyceraldehyde 3-phosphate (Elshafei & Abdel-Fatah 2001, Khosravi *et al.* 2015).

The Leloir pathway requires  $\alpha$ -D-galactose, but the  $\beta$ -anomer is the most common form released during polysaccharides degradation. Aldose 1-epimerase (EC 5.1.3.3), also known as mutarotase epimerizes  $\beta$ -D-galactose into its  $\alpha$ -anomer, which can occur both extracellularly and in the cytosol.  $\alpha$ -D-galactose is phosphorylated by the galactokinase (GalK, EC 2.7.1.6) to form  $\alpha$ -D-galactose 3-phosphate. Galactose-1-

phosphate uridylyltransferase (GalT, EC 2.7.7.12), catalyses the transfer of a UMP group from UDP-glucose to galactose 1-phosphate, generating glucose 1-phosphate and UDP-galactose. To balance the reaction, UDP-galactose is converted to UDP-glucose by UDP-galactose 4-epimerase (EC 5.1.3.2). Finally, phosphoglucomutase (PgmB, EC 5.4.2.2) catalyses the conversion of glucose 1-phosphate into glucose 6-phosphate that enters glycolysis.

GalK (NRRL3\_06978) and PgmB (NRRL3\_05655) are present in all species while GalT (NRRL3\_05970) is only missing in

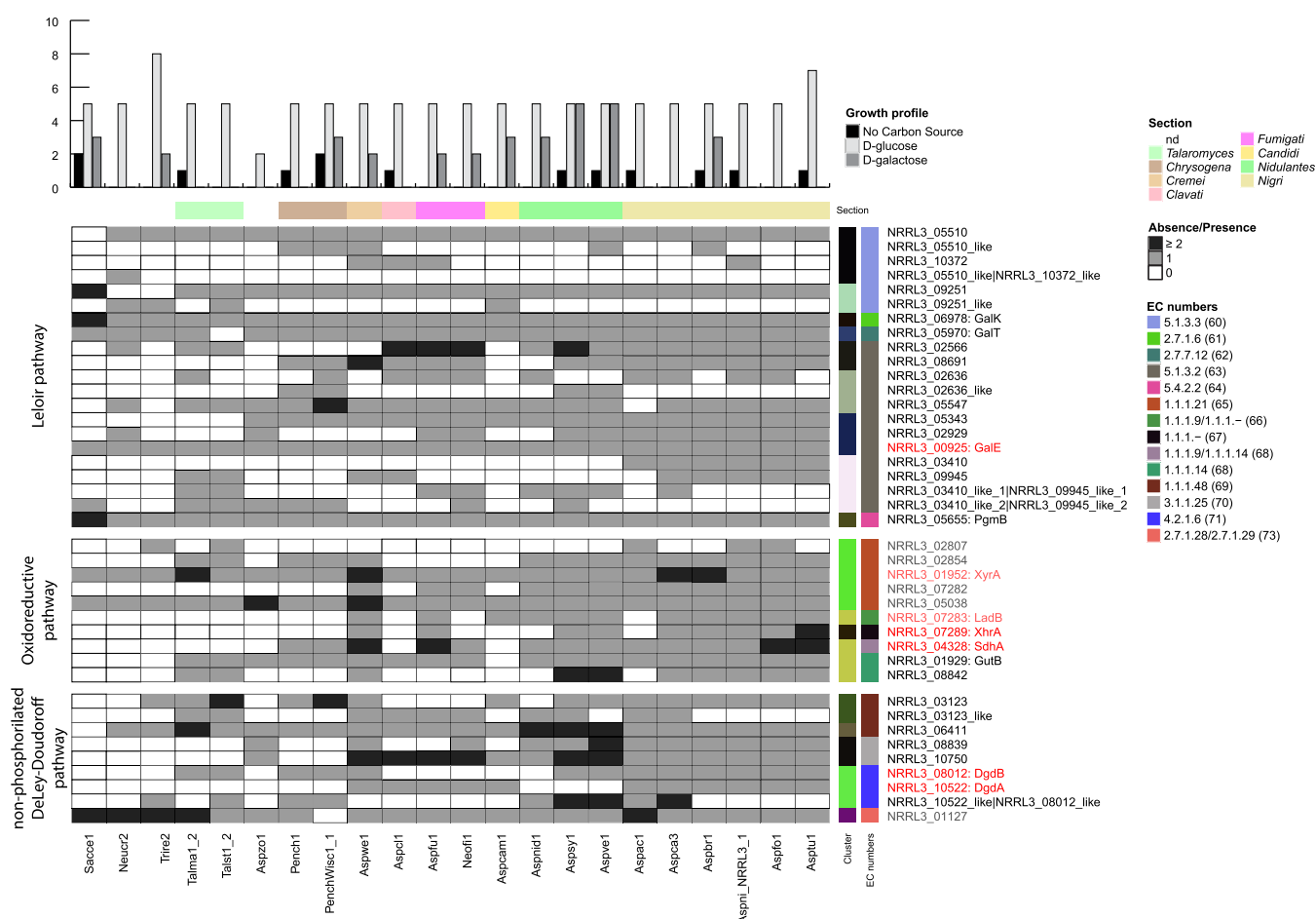


Fig. 9. D-galactose heatmap abundance of *A. niger* orthologous proteins in the D-galactose catabolic pathways. The legends are the same as that of Fig. 3.



*T. stipitatus*. There are two mutarotases predicted, belonging to different clusters, NRRL3\_09251 (intracellular) and NRRL3\_05510 (extracellular), which are both present in all species. A second extracellular mutarotase, NRRL3\_10372 was found through orthology, and is present in *A. wentii*, *A. clavatus*, *A. fumigatus* and *A. niger* exclusively. An ancestral group in this cluster contains an *N. crassa* sequence. Four clusters contain enzymes assigned to UDP-galactose 4-epimerase. GalE (NRRL3\_00925) is the only ortholog present in all species. GalE and the other members of its cluster (NRRL3\_05343 and NRRL3\_02929) are the most conserved orthologs assigned to this activity. Phylogenetic analysis showed that almost every species contains one or more orthologs from other clusters, with the maximum number of proteins in section *Nigri*.  $\beta$ -D-galactose is metabolized directly by the oxidoreductive pathway. Several of the enzymes in this pathway have been shown to also be involved in L-arabinose and D-xylose metabolism (Fekete et al. 2004, Mojzita et al. 2010a,b, Mojzita et al. 2012a,b, Metz et al. 2013). The first step is the reduction of  $\beta$ -D-galactose to galactitol (EC 1.1.1.21). The reduction of the galactitol into L-xylo-3-hexulose is catalysed by the galactitol dehydrogenase (LadB, EC 1.1.1.-) followed by reduction to D-sorbitol catalysed by a reductase specific to D-galactose metabolism, the L-xylo-3-hexulose reductase (XhrA, EC 1.1.1.-). Sorbitol dehydrogenase (SdhA, EC 1.1.1.14) catalyses the conversion of sorbitol to keto-D-fructopyranose which through spontaneous reaction is transformed into its furanose form to enter glycolysis after Hxk transfers a phosphate group to it.

LadB (NRRL3\_07283) and XhrA (NRRL3\_07289) are the only single enzymes assigned in the oxidoreductive pathway, and both appear after *Penicillium* species splits off from the other species. They are missing in *A. clavatus*, *A. fischeri* and *A. aculeatus*. In contrast, there are several oxidoreductases from the same cluster assigned to the production of galactitol (EC 1.1.1.21), but XyrA (NRRL3\_01952) and NRRL3\_05038 are the only orthologs present in all species. The last step of the pathway, includes SdhA (NRRL3\_04328) and GutB (NRRL3\_01929) from two different clusters. GutB is present in all species after *T. reesei* except for *A. campestris*. While SdhA appears after *P. zonata* splits off from the other Eurotiales and is present in all species except *A. clavatus* and *A. campestris*. Neither the *A. niger* NRRL 3 genome functional annotation nor the curated network revealed an aldolase with tagatose-biphosphate affinity suggested in a previous study for the *A. nidulans* genome (Flipphi et al. 2009). This suggests that not all Aspergilli are able to convert D-galactose into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

In the non-phosphorylated DeLey-Duodoroff pathway, the starting substrate is the  $\beta$ -D-galactose form which is oxidized into its lactone form, D-galactono-1,4-lactone by D-galactose dehydrogenase (EC 1.1.1.48). Gamma 1,4 lactonase (EC 3.1.1.25) converts D-galactone 1,4-lactone into D-galactonate, which is further converted by D-galactonate dehydratase (DgdA and DgdB, EC 4.2.1.6) into 2-dehydro-3-deoxy-D-galactonate. Although no aldolase has been associated to the reaction (EC 4.1.2.51), it has been shown (Elshafei & Abdel-Fatah 2001) that the last reaction produces pyruvate and D-glyceraldehyde from 2-dehydro-3-deoxy-D-galactonate. Pyruvate enters the TCA and glyoxylate cycles while D-glyceraldehyde enters glycolysis as D-glyceraldehyde-3-phosphate after a kinase (EC 2.7.1.28) transfers a phosphate group.

Two enzymes from different clusters have been assigned to D-galactose dehydrogenase. NRRL3\_06411 is present in all species,

while NRRL3\_03123 is missing in *N. crassa*, *P. zonata*, *A. clavatus*, section *Fumigati* and *A. nidulans*. A second NRRL3\_03123\_like group that contains sequences of *Talaromyces* species, *A. wentii*, *A. clavati*, section *Fumigati* and some species of sections *Nidulantes* and *Nigri* was found during phylogenetic analysis. A single cluster, contains two  $\gamma$ -1,4-lactonase orthologs (NRRL3\_08839 and NRRL3\_10750) that are present in most species after *Talaromyces*, but *Penicillium* species lack both. Either DgdA or DgdB (NRRL3\_08012 and NRRL3\_10522) is present in all genomes after *T. reesei*, except in *P. zonata* and *A. nidulans*. An *A. nidulans* ortholog is present in a NRRL3\_10522\_like|NRRL3\_08012\_like group as well in other species before *A. campestris*. D-glyceraldehyde 3-phosphate (NRRL3\_01127) is detected in all species except *P. rubens*.

For most of the species that were able to grow on D-galactose, mycelia was used as starting material. In spite of the genomes encoding the enzymes needed for D-galactose metabolism, growth from spores is impossible. Different scenarios need to be considered, transportation inside the cell or lack of induction (Fekete et al. 2012). Except for *A. brasiliensis* which was shown before to be the only *Nigri* able to grow from spores in D-galactose (Meijer et al. 2011). *A. brasiliensis* protein profile differs from the rest of the section *Nigri* in having a close ortholog NRRL3\_05510\_like. This group further contains *A. versicolor*, *A. wentii* and both *Penicillium* species orthologs. With the exception of *P. chrysosporum*, all three species show growth on D-galactose from spores. More studies need to be done to understand the different pathways involved on D-galactose metabolism.

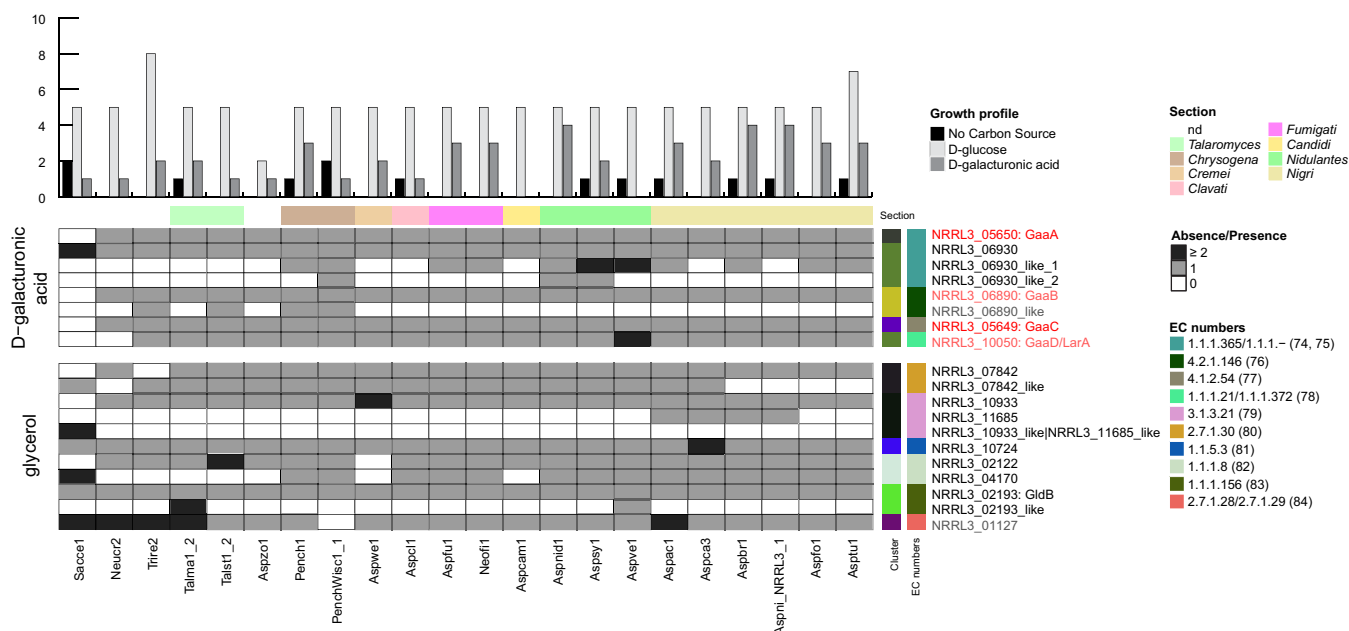
## D-galacturonic acid and L-rhamnose metabolism

D-galacturonic acid and L-rhamnose are both components from pectin and are metabolized through similar but different non-phosphorylated pathways (Alazi et al. 2017, Khosravi et al. 2017). Their recent identification explains why these pathways were not included in the previous inventory of central carbon metabolism in several Aspergilli (Flipphi et al. 2009).

Pectin hydrolysis released D-galacturonic acid monomers that are metabolized through the D-galacturonic pathway (Fig. 10). D-galacturonic acid reductase (GaaA, EC 1.1.1.365/1.1.1.-) converts D-galacturonic acid into aldehyde-L-galactonate. (Alazi et al. 2017), which then is converted by GaaB (NRRL3\_06890, EC 4.2.1.146), encoding a L-galactonate dehydratase, into 2-dehydro-3-deoxy-L-galactonate. 2-keto-3-deoxy-L-galactonate aldolase (GaaC, EC 4.1.2.54) catabolized the reaction splitting 2-keto-3-deoxy-L-galactonate into L-glyceraldehyde and pyruvate. Pyruvate enters TCA and glyoxylate cycles among other pathways, while L-glyceraldehyde is reduced to glycerol by aldolase reductase, (GaaD/LarA, 1.1.1.372). This enzyme has been shown to also be involved in L-arabitol, D-xylositol, D-eritritol and glycerol metabolism (de Groot et al. 2005, Mojzita et al. 2010a, Jovanovic et al. 2013).

All characterised enzymes, GaaA (NRRL3\_05650), GaaB (NRRL3\_06890), GaaC (NRRL3\_05649) and GaaD/LarA (NRRL3\_10050) are present in all studied species. A second enzyme (NRRL3\_06930) assigned to D-galacturonic reductase activity that belongs to a different cluster than GaaA was found in all species, including *S. cerevisiae*. This enzyme could be the responsible for supporting reduced growth observed on D-galacturonic acid when *gaaA* was deleted (Alazi et al. 2017). Several species contain representatives of two NRRL3\_06930\_like groups. Another group was found in the





**Fig. 10.** D-galacturonic and glycerol heatmap abundance of *A. niger* orthologous proteins in the D-galacturonic and glycerol catabolic pathways. The legends are the same as that of Fig. 3.

GaaB cluster (NRRL3\_06890\_like) containing sequences from *Trichoderma reesei*, *Talaromyces stipitatus* and *Penicillium* species.

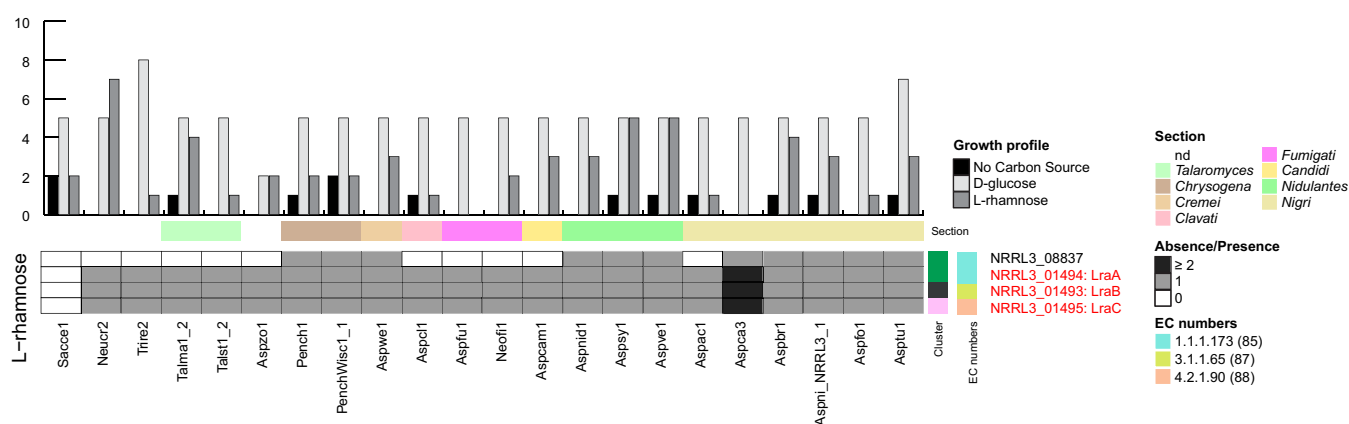
When evaluating growth on D-galacturonic acid, we need to take into consideration glycerol metabolism. Glycerol can be used as carbon source when present in the medium or as an intermediate of other pathways. Glycerol can be converted into sn-glycerol 3-phosphate by the glycerol kinase (EC 2.7.1.30). The reverse reaction is catalysed by glycerol 1-phosphatases (EC 3.1.3.21). Sn-glycerol 3-phosphate is converted into dihydroxyacetone phosphate that enters glycolysis, by two different reversible reactions. A flavin-dependent glycerol 3-phosphate dehydrogenase (EC 1.1.5.3) present in the mitochondrial membrane and a glycerol 3-phosphate dehydrogenases (EC 1.1.1.8). Dihydroxyacetone phosphate can be produced from dihydroxyacetone by a reversible reaction catalysed by glycerone kinase (EC 2.7.1.29). Dihydroxyacetone is produced by the reduction of glycerol catalysed by glycerol hydrogenase (GldB, EC 1.1.1.156).

Glycerol 1-phosphatase NRRL3\_10933, flavin-dependent glycerol 3-phosphate dehydrogenase NRRL3\_10724 and GldB (NRRL3\_01127) are present in all species. Glycerol kinase

NRRL3\_07842 is also present in all species except *S. cerevisiae* and *T. reesei*, and its ortholog group NRRL3\_07842\_like is only missing in section *Nigri* and *N. crassa*. Besides NRRL3\_10933, another phosphatase has been found in the same cluster (NRRL3\_11685), which is only present in some species from section *Nigri*. The *A. wentii* genome lacks all glycerol 3-phosphatase candidates (NRRL3\_02122 and NRRL3\_04170), while the rest of the genomes contain at least one of the orthologs. Glycerol metabolism in *S. cerevisiae* is an important source of energy, and its genomes contains at least one copy of each enzyme.

With the exception of *P. rubens*, *A. clavatus*, *A. campestris* and *A. versicolor*, all species are able to grow on D-galacturonic acid as sole carbon source with no difference related to genome content. Therefore, we can assume, that glycerol metabolism is also functional in the rest of the species.

L-rhamnose is released from polysaccharides such as pectin or rhamnogalacturonan type I in its  $\beta$ -pyranose form. Similar to D-galacturonic acid, L-rhamnose is transformed into the furanose form through a spontaneous reaction to enter the L-rhamnose degradation pathway (Fig. 11). L-rhamnose is converted into L-rhamnose-1,4-lactone by a NADH-dependent L-rhamnose-1-



**Fig. 11.** L-rhamnose heatmap abundance of *A. niger* orthologous proteins in the L-rhamnose catabolic pathways. The legends are the same as that of Fig. 3.

dehydrogenase (LraA, EC 1.1.1.173) (Khosravi et al. 2017). The L-rhamnonic acid lactonase (LraB, EC 3.1.1.65), catalyses the hydrolysis of L-rhamnono-1,4-lactone to L-rhamnonate, while L-rhamnonate dehydratase (LraC, EC 4.1.2.53) converts this into 2-dehydro-3-deoxy-L-rhamnonate. The enzyme catalysing the last step in the pathway, an aldolase (EC 4.2.1.53) remains to be discovered.

All species contain one copy of each known Lra enzyme, LraA (NRRL3\_01494), LraB (NRRL3\_01493) and LraC (NRRL3\_01495), except *A. carbonarius* that contain more than one copy of all three. A second paralog of LraA, NRRL3\_08837 was found in the same cluster and is present in all species after *P. zonata* except *A. clavatus*, *A. campestris*, *A. aculeatus* and section *Fumigati*.

A similar phenotype was observed in species belonging to different clades. Those species that grow on L-rhamnose show either reduced or similar growth to D-glucose, except *N. crassa* which shows better growth than on D-glucose. Interestingly, *A. carbonarius* was not able to grow on L-rhamnose even though it has extra copies of all known Lra genes.

## Pentose catabolism

Pentose catabolism in *A. niger* mainly involves two pathways, the pentose catabolic and pentose phosphate pathways. The pentose catabolic pathway includes L-arabinose and D-xylose catabolism to produce D-xylulose, and the pentose phosphate pathway includes D-ribulose, D-ribose and D-xylulose catabolism and the production of several glycolytic intermediates including NADPH necessary during glycolysis (Fig. 12).

In the first step in L-arabinose metabolism, L-arabinose reductase converts L-arabinose into L-arabitol (GaaD/LarA, EC 1.1.1.-, 1.1.1.21) (de Groot et al. 2005, Mojzita et al. 2010a, Jovanovic et al. 2013). L-arabitol oxidation to L-xylulose is catalysed by L-arabitol 4-dehydrogenase (LadA, EC 1.1.1.12) (Kim et al. 2010, Mojzita et al. 2012b). The last step is the reduction of L-xylulose to produce xylitol by xylitol dehydrogenase (LxrA, EC 1.1.1.10).

D-xylose conversion into xylitol is catabolized by D-xylose reductase (XyrA, EC 1.1.1.21). The final product of the separate L-arabinose and D-xylose pathways, xylitol, is reduced to D-xylulose by xylitol dehydrogenase (EC 1.1.1.9), which is phosphorylated by D-xylulose kinase to form D-xylulose 5-phosphate (XkiA, 2.7.1.17).

D-xylulose 5-phosphate originates from the pentose catabolic pathway and is one of the main substrates of the pentose phosphate pathway. Glycolysis connects to the pentose phosphate pathway through D-glucose 6-phosphate conversion to 6-phospho D-gluconate-1,5-lactone by the glucose-6-phosphate 1-dehydrogenase (GsdA, EC 1.1.1.49) (van den Broek et al. 1995). 6-phosphogluconolactonase (EC 3.1.1.31) hydrolyzes 6-phospho D-glucono-1,5-lactonate to produce D-gluconate 6-phosphate, which is further catabolized by 6-phosphogluconate dehydrogenase (EC 1.1.1.44) to D-ribulose 5-phosphate. The D-gluconate pathway also connects to the pentose phosphate pathway through D-gluconate 6-phosphate.

D-ribulose 5-phosphate can be produced from the degradation of D-ribulose catalysed by ribulokinase (RbtA, EC 2.7.1.47), and is then converted by ribulose-phosphate 3-epimerase (RpeA, EC 5.1.3.1) to D-xylulose 5-phosphate or by ribose 5-phosphate isomerase (Rpi, EC 5.3.1.6) to D-ribose 5-phosphate. Transketolase (TktA, EC 2.2.1.1) catalyses the reaction between D-ribose 5-phosphate and D-xylose 5-phosphate

to produce D-sedoheptulose 7-phosphate and D-glyceraldehyde 3-phosphate. The latter can enter glycolysis or be utilized by transaldolases (Tal, EC 2.2.1.2) to produce D-erythrose 4-phosphate and  $\beta$ -D-fructofuranose 6-phosphate that can also enter glycolysis or other pathways. Transketolase (TktA), can also convert D-erythrose 4-phosphate and D-xylose 5-phosphate into  $\beta$ -D-fructose 6-phosphate and D-glyceraldehyde creating a loop in the pathway. A different transketolase (TktB, EC 2.2.1.3) catalyses the production of D-glyceraldehyde and dihydroxyacetone from D-xylulose 5-phosphate and formaldehyde.

Except for XkiA (NRRL3\_04471) that is present in all species, all reactions in the pentose catabolic pathway have multiple enzymes assigned. Five enzymes have been assigned to L-arabitol and xylitol production. Through phylogenetic analysis, we found a total of eight proteins predicted to be involved in those reactions, but only two have been biochemically characterised GaaD/LarA and XyrA (Hasper et al. 2000, Jovanovic et al. 2013). *In vitro* analysis showed activity of GaaD/LarA and XyrA on L-arabinose, D-xylose and for the latter on D-galactose as well, but growth on L-arabinose or D-xylose is already reduced when LarA or XyrA are missing (Mojzita et al. 2010a). GaaD/LarA (NRRL3\_10050) shows the highest affinity for L-arabinose and L-arabitol while XyrA (NRRL3\_01952) shows higher affinity for D-xylose and xylitol compared to other enzymes. Orthologs of both genes can be found in all studied species. The NRRL3\_05038 ortholog is also found in all species. Interestingly, both XyrA and NRRL3\_05038 are the only enzymes for these reactions that are also present in *S. cerevisiae* and *N. crassa*. The other genomes contain different orthologs for several enzymes, up to eight in some species from section *Nigri*. LadA (NRRL3\_02523) is present in all species but its ortholog NRRL3\_00896 is missing in *N. crassa*, *T. stipitatus*, *P. zonata*, *A. clavatus* and *A. campestris*. Those species together with *Trichoderma reesei*, *Talaromyces marneffeii*, *Penicillium* species, *A. campestris* and sections *Fumigati* and *Nidulantes* also miss NRRL3\_08407 in their genome. LxrA (NRRL3\_10884) is the only xylitol dehydrogenase characterised and it is present in all species. From the same cluster, NRRL3\_04510 is missing in the genome of *A. campestris*. Species from section *Nidulantes* contain all four orthologs.

Several enzymes have been assigned to D-xylulose production but only some of them have been biochemically characterised. XdhA (NRRL3\_09204) shows the highest activity with xylitol and D-xylulose, while LadA (NRRL3\_02523) is also active on xylitol and D-xylulose but has higher activity on L-arabitol and L-xylulose (de Groot et al. 2007). XdhA, LadA and NRRL3\_00319 are present in all species. From the same cluster, five additional orthologous groups were identified. *A. wentii*, *A. versicolor*, *A. brasiliensis* and *A. niger* contain representatives of all eight groups.

GsdA (NRRL3\_05283), RbtA (NRRL3\_00588), RpeA (NRRL3\_00279), RpiA (NRRL3\_10107) and TktA (NRRL3\_11249) are present in all species, while PgiA (NRRL3\_02043) is missing in *A. campestris*. Two different clusters contain five enzymes with 6-phosphogluconate dehydrogenase activity (EC 1.1.1.44), which were found through phylogenetic analysis. Only NRRL3\_09981 is present in all species, including *S. cerevisiae*. Most of the species contain also NRRL3\_09641 and NRRL3\_09981 orthologs, except for *T. reesei*, but NRRL3\_08784 is only present in *A. wentii*, *A. sydowii*, *A. versicolor*, *A. aculeatus* and *A. niger*.

Ribose 5-phosphate isomerase assigned enzymes (RpiA: NRRL3\_10107 and RpiB: NRRL3\_06024) belong to different clusters. RpiB is missing in *A. fumigatus*, while NRRL3\_06024-like group only contains orthologs from *A. wentii*

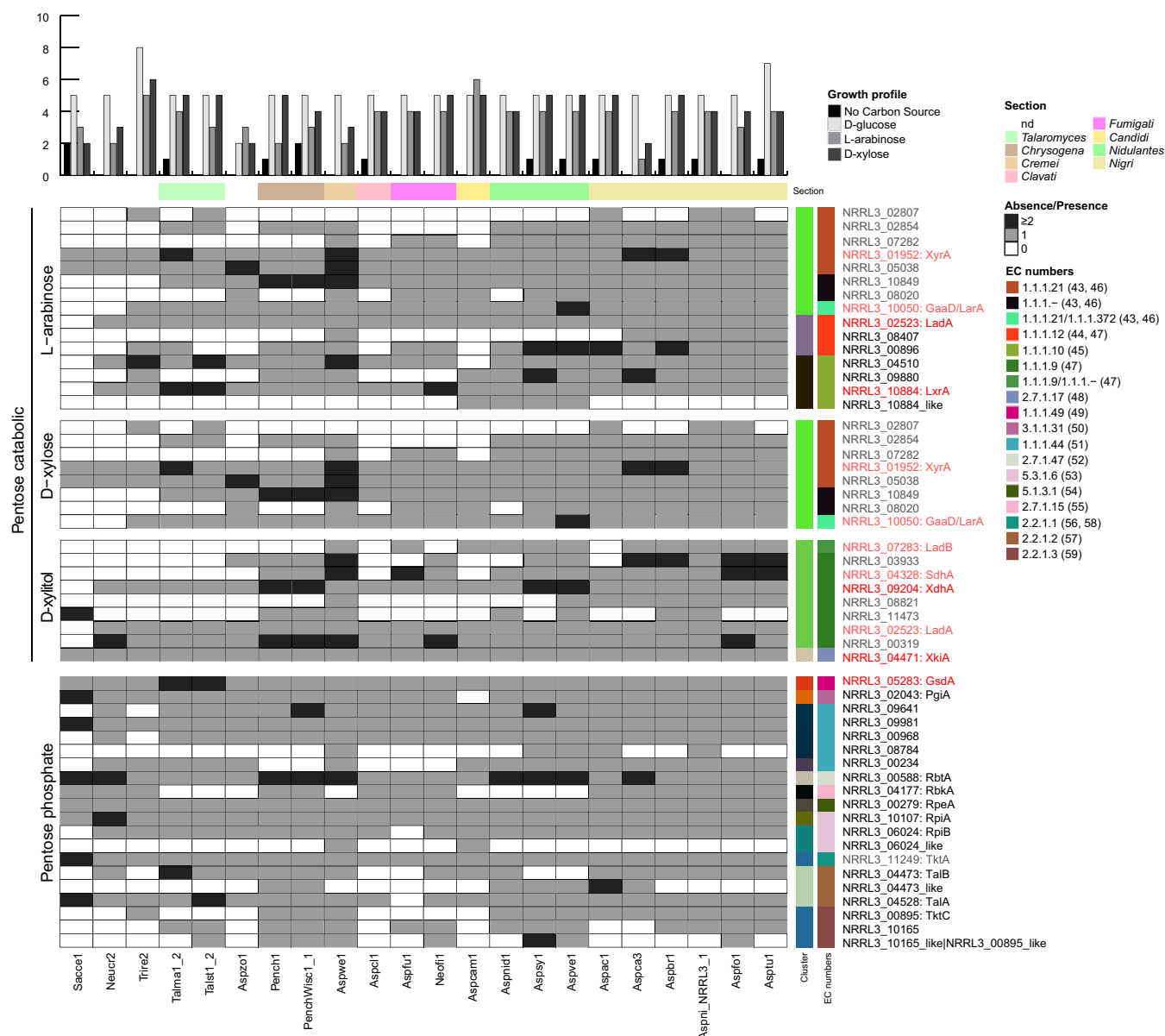


Fig. 12. Pentose metabolism heatmap abundance of *A. niger* orthologous proteins in the pentose metabolic pathways. The legends are the same as that of Fig. 3.

and *A. campestris*. There are two transaldolases predicted (TalA, NRRL3\_04528 and TalB, NRRL3\_04473) that belong to the same cluster. TalA is missing in *A. fumigatus* while TalB is missing in *T. reesei*, *A. clavatus*, *A. fumigatus* and some species from section *Nidulantes*. This implies that there is no transaldolase similar to these genes in *A. fumigatus*, which implies that a non-homologous gene is responsible for this function or that the gene has not been identified or assembled in the *A. fumigatus* genome. In contrast, NRRL3\_04473\_like orthologs are only present in *A. aculeatus*, *A. carbonarius*, section *Nidulantes* and *Penicillium* species. Dihydroxyacetone synthase (EC 2.2.1.3), TktC (NRRL3\_00895) and NRRL3\_10165 belong to the same cluster as TktA (NRRL3\_11249, EC 2.2.1.1). They have been assigned a different reaction in the network. They catalysed the production of dihydroxyacetone and D-glyceraldehyde 3-phosphate from D-xylulose 5-phosphate and formaldehyde. Only *Penicillium* species, *A. versicolor* and *A. luchuensis* contain all three orthologs predicted, most species contain only two orthologs in their genomes.

All species were able to grow on L-arabinose and D-xylose to different degrees. Interestingly, even though both *Penicillium*

species have the same pattern (except extra copies of NRRL3\_09641) they behaved differently. *P. chrysogenum* grew on D-xylose as good as on D-glucose while *P. rubens* showed better growth on L-arabinose but always lower than on D-glucose. *A. campestris* grew better than any other species on L-arabinose compared to D-glucose, despite missing several orthologous enzymes for most of the activities. Yeasts like *S. cerevisiae* are known for being unable to grow on pentoses unless genetically modified (Jeffries 2006, Hahn-Hagerdal et al. 2007). As shown in the Fig. 12, they miss most of the orthologous proteins involved exclusively in pentose catabolic metabolism, but not pentose phosphate metabolism that is needed to produce NADPH for glycolysis.

## DISCUSSION

Automated pipelines for genome assembly, gene prediction and functional annotation have evolved over the years to handle increasingly larger next generation sequencing output files. At the same time, human supervision has reduced compared to the initial genomes. In our protein assessment analysis, we show

how gene prediction quality varies between genomes regardless of organism, technology or consortia. Comparing the predicted proteome against a database of conserved proteins enables evaluation of the completeness of the proteome and therefore the quality of the gene annotation and genome assembly. Different options can be used to improve a new genome. The most common option is to use a similar genome from a closely related species to improve gene prediction and functional annotation, but this only provides significant improvement if that genome is of high quality. However, not many high-quality genomes are available. Gold-standard genomes are difficult to acquire, they require a high amount of omics data and manual curation by experts at every stage of the process. We showed in this study that more than 90 % of predicted proteins in genus *Penicillium* shared sequence similarity with proteins from genus *Aspergillus*. Using a gold-standard genome can improve gene prediction and protein functional annotation and, if combined with metabolic network analysis, it can be used to predict metabolic pathways with higher confidence. This analysis goes significantly beyond the use of the KEGG automatic pipeline together with orthology analysis and a partially completed genome sequence that was used in the previous study based on *A. nidulans* (Flipphi et al. 2009).

In this study, we also showed how orthology and protein profiling based on a manually curated metabolic network of a gold-standard genome (*A. niger* NRRL 3) can be used to predict primary carbon metabolism in a number of species and correlate the genome content with growth abilities. As expected, more distant Ascomycota, *S. cerevisiae*, *N. crassa* and *T. reesei*, contain lower number of orthologs of *A. niger* NRRL 3 genes compared to other members of the genus *Aspergillus*. In particular, *S. cerevisiae* lacks several proteins and even complete pathways compare to *A. niger* NRRL 3.

Glycolysis operates with remarkable efficiency if we consider the number of pathways that link to glycolysis through D-glucose 6-phosphate and D-fructose 6-phosphate. Some of the steps of the pathway have been associated with several enzymes, but we find less redundancy in key enzymatic reactions, especially in the steps leading to production of pyruvate from D-glyceraldehyde 3-phosphate. Species from section *Nigri* are known for citric acid production (Max et al. 2010), which correlates with the high number of orthologous genes assigned to the glyoxylate and TCA cycles. A similar pattern is observed for the genes involved in D-gluconate production from D-glucose, which also correlates with previous data showing that *Penicillium* and *Aspergillus* section *Nigri* species are high gluconate producers (de Vries et al. 2017).

A clear example of the presence of an alternative pathway is growth of *S. cerevisiae* on D-gluconate (Oliva Neto et al. 2014), while our study indicates that its genome misses all the enzymes of the pathway. Another example of alternative mechanism occurs during growth on sucrose. *S. cerevisiae*, *N. crassa*, *P. zonata*, *A. clavatus* and *A. campestris* genomes lack all three GH32 proteins identified in *A. niger*, even though they can grow on sucrose almost as well as on D-glucose and D-fructose. This suggests that these species contain a non-homologous alternative enzyme that can split sucrose.

In contrast, the *A. clavatus* genome contains genes that encode for all identified enzymes necessary for D-galacturonic catabolism, but it is not able to grow on D-galacturonic acid as a sole carbon source. A possible explanation for this is the absence of the galacturonic acid transporter in *A. clavatus*

(Mäkelä et al. 2018). Tight regulation on the transporter seems to be causing the lack of growth from spores on D-galactose as sole carbon source (Fekete et al. 2012). Even though most species contain orthologs for the *A. niger* genes, most of them are still unable to grow from spores, similar to what is observed for *A. niger* itself. They are able to grow when small amounts of other monosaccharides are present in the media or when they are inoculated from mycelia, which supports that the problem is mainly related to uptake of D-galactose during germination.

In summary, we show that combining a gold-standard genome with a manual curated metabolic network and phylogeny can significantly improve functional annotation of less studied species. It helps with the identification of essential proteins and alternative pathways for the degradation of several compounds that otherwise could have been missed or required significant efforts through classical genetics. We observed low diversity between closely related species for monosaccharide catabolism, and even high conservation between more distant fungi. This is likely due to the important role monosaccharides have as a carbon source for fungi. We can expect a higher diversity between species for other aspects of fungal biology, such as in polysaccharide degradation, transport affinity and regulatory systems. In fact, other papers in this issue reveal high diversity with respect to plant biomass degradation (Mäkelä et al. 2018), stress tolerance (Emri et al. 2018) and sexual and asexual reproduction (Ojeda-López et al. 2018).

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.simyco.2018.10.001>.

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